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Osmoregulation and FMRFamide-Related Peptides in the Salt Marsh Snail *Melampus bidentatus* (Say) (Mollusca: Pulmonata)

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Abstract. The pulmonate snail *Melampus bidentatus* occupies the high intertidal zone of salt marshes in a nearly terrestrial environment. The hemolymph osmolarity of the snails collected in the field paralleled that of the adjacent water and was affected by the tides and precipitation. The snails initially gained or lost weight when submerged in hypo- or hyperosmotic media, respectively, but returned to their original weight after 24 h. The content of their immunoreactive (IR)-*F*MRF*a*mide-*R*elated *P*eptides (FaRPs) was measured in various tissues by radioimmunoassay, and IR-FaRPs were found in every tissue analyzed. The subesophageal part of the central nervous system (CNS) contained more IR-FaRPs than the supraesophageal part, and the kidney and the tissues of the reproductive tract contained more than other peripheral tissues. The levels of IR-FaRPs in the CNS, kidney, and hemolymph were higher in snails that were immersed in higher concentrations of seawater. Many IR neurons are present in all ganglia of the CNS except the pleural ganglia, and IR neurites are extensively distributed within the CNS and its connective tissue sheath. The visceral nerve from the visceral ganglion is immunoreactive and could be seen to innervate the kidney, which contains IR-varicosities. An osmoregulatory role for the FaRPs is suggested.

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Abbreviations: One-letter abbreviations of the amino acids are used to state the peptide sequences. ASW, artificial seawater; BSA, bovine serum albumin; CNS, central nervous system; FaRPs, *F*MRF*a*mide-*r*elated *p*eptides; IM, incubation medium; IR, immunoreactive; NGS, normal goat serum; PBS, phosphate-buffered saline; RIA, radioimmunoassay.

Introduction

Melampus bidentatus is a common amphibious pulmonate snail. Its habitat is the high intertidal zone of salt marshes, and it ranges from Nova Scotia, Canada, to the coast of the Gulf of Mexico in Texas (Apley, 1970; Hilbish, 1981). *Melampus* belongs to the primitive family Ellobiidae and is believed to be related to an ancestral marine strain that colonized the intertidal habitat, giving rise to the land snails and then to the freshwater snails (Morton, 1955; Russell-Hunter, 1978). Environmental factors such as temperature, salinity, and tides are extremely variable in the high littoral habitat. But the adult snails are adapted to this variation, and can survive for several days at temperature extremes of −12°C to 40°C, submergence in 25%–100% seawater, and desiccation at 0% relative humidity for 27–36 h (Price, 1980; McMahon and Russell-Hunter, 1981). Although adults of *M. bidentatus* can endure a terrestrial life, its planktonic veliger larvae are restricted to the aquatic habitat of estuaries (Russell-Hunter *et al.*, 1972).

The natural history of *M. bidentatus* suggests that in estuarine and semiterrestrial molluscs, osmotic and volume regulation must be particularly responsive. Moreover, scattered evidence suggests that the family of neuropeptides related to FMRFamide is involved in this regulation. First, in a freshwater pulmonate snail, *Helisoma duryi*, FMRFamide causes water retention in the kidney *in vitro* (Saleuddin *et al.*, 1992). Further, the level of immunoreactive, FMRFamide-related peptides (IR-FaRPs) in the kidney of *Helisoma trivolvis* kept in hypoosmotic medium is lower than that in the kidneys of snails kept in isosmotic medium (Madrid *et al.*, 1994). The IR-FaRPs have also been localized in the kidneys of the terrestrial pulmonate snail *Helix*

aspersa (Lehman and Price, 1987), and in the central nervous system (CNS) of the veligers of *M. bidentatus* (Moffett, 1992). Finally, the regulation of hypoosmotic cell volume by cardiac muscles of the clam *Mercenaria mercenaria* is potentiated by FMRFamide (Deaton, 1990).

Since *M. bidentatus* inhabits highly variable osmotic conditions, it is an appropriate species with which to further test the proposal that the FaRPs have a role in osmoregulation. In this paper, we have studied, both in the field and the laboratory, the influence of the ambient osmotic concentration on that of the hemolymph. We have then compared the levels of IR-FaRPs in the CNS, hemolymph, and kidney of snails maintained in media of different osmotic concentrations. We also report IR-FaRP-staining of neurites in the kidney, and of neurons in the CNS of adult snails.

Materials and Methods

Animals and media

During January to April, *M. bidentatus* adults (shell lengths, 10.0 ± 2.0 mm) were collected at random from an area 100 m × 20 m in a salt marsh on the Matanzas river estuary near Crescent Beach, Florida (29°40′ N : 81°13′ W). Experiments with the freshly collected snails were performed nearby, at the Whitney Laboratory of the University of Florida. At York University, the animals were maintained at 22°C, 95% relative humidity, and a photoperiod of 14 h light to 10 h dark, in glass tanks (30 cm L × 15 cm W × 20 cm H) covered with nylon screens. The floor of each tank was covered with a layer of crushed oyster shells (poultry feed) graded in thickness from 0.5 cm at one end of the tank to 3.0 cm at the other. A volume of 50% artificial seawater (ASW; Instant Ocean, Aquarium Systems, Ohio) was added to cover about one-third of the crushed oyster shells. Slanted against the walls of each tank were 4–6 broken, irregularly shaped pieces of clay plant-pot (about 10 cm in diameter). Most snails crawled to terrestrial conditions above the water level on the walls of the tank and on the dark side of the moist pieces of clay plant-pot. Distilled water was added daily to maintain osmolarity and water level, and the snails were fed fish-food and boiled lettuce *ad libitem*. The snails were acclimated to laboratory conditions for at least 2 weeks before experimentation.

The kidney is embedded just under the dorsal surface of the mantle tissue, and the CNS is located under the skin, between the tentacles. Before dissection, the snails were chilled on ice for 5–10 min, which reduced their movements and mucus secretion; no other anesthetic was used. Deshelled snails were pinned on a dish lined with Sylgard (Dow Corning, Canada) and dissected in filtered isosmotic ASW. We adjusted the osmolarity of the ASW with distilled water to be isosmotic with that of the hemolymph, which varies with the medium or relative humidity (described later). The kidney and CNS were removed from a snail within 3–5 min, and were used for FaRP studies.

Osmotic conditions

Snails that had been maintained under terrestrial conditions (described above) were submerged for various periods (maximum, 4 days; minimum 3 h) in 10%, 50%, or 100% ASW (dilutions made with distilled water) in 250-ml plastic containers, each with a nylon screen. The levels of IR-FaRPs were measured (described later) after 24 h of osmotic treatments. Hemolymph was collected as follows. The snails were pricked in the head area with a sharp needle. Each animal was then quickly placed in a 0.5-ml polypropylene microcentrifuge tube with a hole in its bottom. This tube was then placed in a larger (1.5 ml) polypropylene microcentrifuge tube. The nested tubes and the snail were spun at 500 × *g* for 2–5 s. Then the inner tube containing the snail was removed, and the outer tube containing the hemolymph was spun for an additional 5 min. About 30–50 μl of hemolymph could be collected from each snail. The osmotic pressures of 10-μl samples were measured in a Wescor Model 5300 vapor pressure osmometer (Logan, Utah). After the bleeding, all of the snails fed and lived for many days thereafter. To measure the effects of osmotic water exchange on weight, the snails were weighed with a Mettler AE163 balance after the visible water and mucus had been removed from their shells and feet with adsorbent tissue.

Radioimmunoassay

Pulmonate molluscs contain two major classes of FaRPs: the tetra-FaRPs (FMRFamide and FLRFamide) and the hepta-FaRPs (XDP[F/Y]LRFamide, where the N-terminal residue X is pQ, S, N, or G). The hepta-FaRP analog with the glycyl residue occurs only in snails of the subclass Basommatophora (like *M. bidentatus*), and these animals lack the analog with the pyroglutamic acid (pQ) residue, which occurs only in stylommatophorans (Price *et al.*, 1987a, b). To ensure that all of the FaRPs would be detected, we used S253 antiserum in the radioimmunoassay (RIA). The antiserum was raised to thyroglobulin-peptide (synthetic analog YGGFMRFamide) conjugate in a rabbit. It has high affinities for both FMRFamide and GDPFLRFamide, and was used in the assays at a dilution of 1:10,000. Iodinated pQYPFLRFamide was used as tracer (for details see: Price *et al.*, 1990; Lesser and Greenberg, 1993).

The protocol of Madrid *et al.* (1994) was followed in processing the tissues. After dissection, each tissue sample was placed in a plastic tube (1.5 ml) containing HPLC grade acetone (1 part tissue: 4 parts acetone) and frozen immediately at −80°C for at least 24 h. The acetone extracts were spun for 5 min at 5,000 × *g*, and the supernatants were collected and then dried in a Speed-Vac centrifuge. Each

pellet was dissolved in 100 μl RIA buffer [0.01 *M* sodium phosphate with 1% bovine serum albumin (BSA), 0.9% sodium chloride, 0.01% merthiolate, and 0.025 *M* sodium EDTA]. Aliquots of 10 μl were placed in glass tubes to which 100 μl tracer (10,000 cpm) (in RIA buffer) and 100 μl diluted antiserum (in RIA buffer) were added. The tubes were stored at 4°C overnight; on the following morning, 1.0 ml charcoal suspension (0.25% charcoal, 0.025% dextran, 0.01% merthiolate in 0.1 *M* sodium phosphate, pH 7.5) was added to each tube. After 10 min, the mixture was centrifuged at 2500 × *g* for 15 min at 4°C, and the supernatant was counted in an LKB MiniGamma counter. All statistical comparisons were made with a one-way analysis of variance (ANOVA); when the ANOVA showed a significant difference, the effect was followed with the Bonferroni multiple comparison post test using a statistical software package (InStat, GraphPad Software, San Diego, California).

Immunocytochemistry

Isolated CNS and kidney tissues from animals in laboratory terrestrial condition were either embedded in paraffin and sectioned, or examined in whole mount. Sections were prepared as follows. The tissues were fixed for 12–18 h in modified Bouin-Hollande [7% picric acid, 2.5% copper acetate, 2% formaldehyde (freshly prepared from paraformaldehyde), and 1.5% glacial acetic acid, in phosphate-buffered saline (PBS) (0.2 *M* NaCl, 0.003 *M* KCl, 0.002 *M* KH_2PO_4, 0.02 *M* Na_2HPO_4, 0.001 *M* $CaCl_2$, 0.001 *M* $MgCl_2$, pH 7.5)], then dehydrated in graded ethanol solutions and embedded in paraffin. Serial sections (10 μm) were cut and mounted on coverslips (22 mm × 22 mm) coated with 0.5% gelatin, 0.5% chrome alum, and 0.01% formaldehyde. The deparaffinized sections were treated with 100% methanol and 0.1% H_2O_2 for 5 min, hydrated in a graded series of ethanol solutions, kept 30 min in an incubation medium (IM) consisting of PBS containing 2% Triton × 100 (Sigma Chem., St. Louis, MO), 5% normal goat serum (NGS), and 1% BSA. The sections were incubated for 1 h at 22°C with hepta-FaRP-specific CK antiserum, which was raised in a rabbit to the peptide CKQDPFLRFGK (a gift from Dr. G.A. Cottrell, University of St. Andrews, Scotland) (Cottrell *et al.*, 1994). The primary antiserum was diluted 1:200 in IM. After many rinses in IM for 10 min, the sections were incubated for 1 h at 22°C in fluorescein-conjugated goat antirabbit-serum (Sigma) diluted 1:80 in IM (secondary antibody). The coverslips were rinsed well in PBS and mounted on glass slides in 5% polyvinyl alcohol, 30% glycerol, and 0.1% phenylenediamine in PBS (mounting medium).

Whole mounts were prepared as follows. The tissues were treated with proteases before and after fixation (Longley and Longley, 1986). The tissues were first treated in 0.05% pronase in isosmotic saline for 10 min, then fixed in Bouin-Hollande (see above) for 12 h. After fixation, the tissues were rinsed in saline for 10 min, then treated in 0.1% trypsin in saline for 30 min, and rinsed well in saline for 10 min. A preincubation in IM for 6–12 h at 4°C was followed by incubation in primary antibody (hepta-FaRP-specific CK antiserum; 1:200 in IM) for 24 h. After rinsing several times in 1% Triton X 100 in PBS for 1 h, the tissues were incubated in secondary antibody (1:80 in IM) for 6 h, followed by repeated rinses in IM. When whole-mount tissues were incubated for more than 60 min, this was done in the dark at 4°C. The tissues were mounted in mounting medium (see above) between two coverslips (one rectangular, 60 mm × 22 mm; and one circular, 18 mm). To prevent tissue distortion, the weight of the coverslip was supported by four pieces of broken coverslip (~0.5 mm diameter). The larger coverslip was affixed with adhesive tape on an aluminum slide (36 mm × 80 mm × 1 mm) with a central 25-mm circular window. The smaller circular coverslip was placed face down in this window, so these preparations could be flipped for viewing from either side.

For controls, either pre-immune rabbit serum (1: 200 in IM) or IM alone was used in the primary incubation; in either case, the tissues were then stained with secondary antibody as described above. The controls showed no staining. The specimens were viewed and photographed with either a Leitz epifluorescence microscope or a Bio-Rad MRC 600 confocal microscope. The IR-FaRP cells were mapped from the serial sections and from whole-mount images, and were measured with the calibration marker from the confocal images.

Results

Osmoregulation

The high-tide location of the marsh where *M. bidentatus* was collected has an uneven surface with a shallow slope, and contains abundant decaying organic matter of plant and animal origin. The osmolarity and pH of the waters in the habitat varied with time and location in the observation area. During the sunny days of April, the average osmolarities were 1200 ± 26 mosm/kg H_2O (mean ± standard error of mean, $n = 50$) (range = 1015–1650 mosm/kg H_2O), and the pH ranged from 6.0–7.2; at 1200–1400 h, the ambient air temperature was 31 ± 4°C, and the soil surface and water temperatures were 34.2 ± 1.3°C. At the same time, the tidal seawater coming through the Matanzas inlet had a consistent osmolarity of 980 ± 5 mosm/kg H_2O, a temperature of 22 ± 2°C, and a pH of 7.5 ± 0.1. The variables that altered the osmolarities of the water in the habitat and hemolymph of snails were tide, temperature, and precipitation. Most snails in the field were out of the water, on grass stems or higher points of the uneven ground, while a much smaller number were creeping and feeding underwater. The osmo-

larities of the hemolymph of snails from different locations and conditions and during high and low tides were measured within 30 min after collection and compared with the osmolarities of the adjacent media. The hemolymph osmolarities ranged from 1100 to 1500 mosm/kg H_2O and appeared to parallel that of the adjacent medium. A few hours of heavy rainfall reduced the osmolarity of the water in the habitat to 750 ± 40 mosm/kg H_2O; the hemolymph osmolarities also declined to 850 ± 50 mosm/kg H_2O. Most snails in the laboratory crawled to the dry parts of the tank (see methods section) and stayed away from the water; thus they sought terrestrial conditions. But a few snails were also seen feeding and crawling underwater. The hemolymph osmolarity of the laboratory snails that sought terrestrial conditions was much lower (450 ± 25 mosm/kg H_2O) than that of field snails (1200 ± 100 mosm/kg H_2O) collected away from the water. When laboratory snails from terrestrial conditions were held for 24 h in 10% or 50% ASW, the osmolarity of the hemolymph paralleled, and was about 150 mosm/kg H_2O hyperosmotic to, that of the medium. Snails in 10% and 50% ASW gained weight during the first 3–6 h, but over 24 h, gradually returned towards their original weight. In contrast, snails kept in 100% ASW for 24 h first lost weight and then returned towards their original weight (Fig. 1).

Levels of IR-FaRPs

Immunoreactive-FaRPs were detected in every tissue studied; the CNS had higher levels than the other organs. The supraesophageal portion of the CNS (the buccal and cerebral ganglia) had lower levels of IR-FaRPs than the subesophageal portion (the pedal, parietal, and visceral ganglia) (Table I).

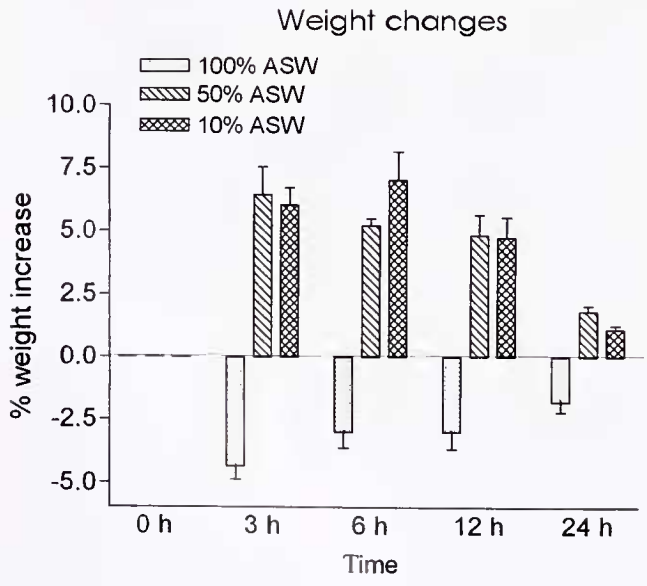


Figure 1. Time course of weight changes of snails acclimated under laboratory conditions and subsequently submerged in 10%, 50%, and 100% ASW (n = 10; mean ± standard error of mean).

Table I.

Immunoreactive FaRP content detected by S253 antiserum in various tissues of animals from terrestrial condition in laboratory

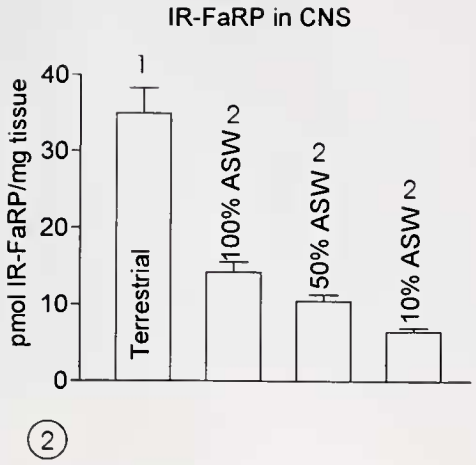
Tissue	IR-FaRP content (picomol)
Buccal ganglia*	1.2 ± 0.40a
Cerebral ganglia*	4.0 ± 0.70a
Pedal ganglia*	19.1 ± 7.0 b
Visceral and parietal ganglia*	13.50 ± 6.0 b
Heart*	1.07 ± 0.40a
Hemolymph (50 μl)	0.35 ± 0.10a
Buccal mass	0.20 ± 0.07a
Foot	0.22 ± 0.09a
Digestive gland	0.36 ± 0.08a
Intestine	0.12 ± 0.03a
Kidney	3.45 ± 0.36a
Mantle	1.28 ± 0.20a
Ovotestis	1.10 ± 0.60a
Penis complex	3.55 ± 0.08a
Skin	0.40 ± 0.20a
Vulva/vagina complex	4.20 ± 2.00a

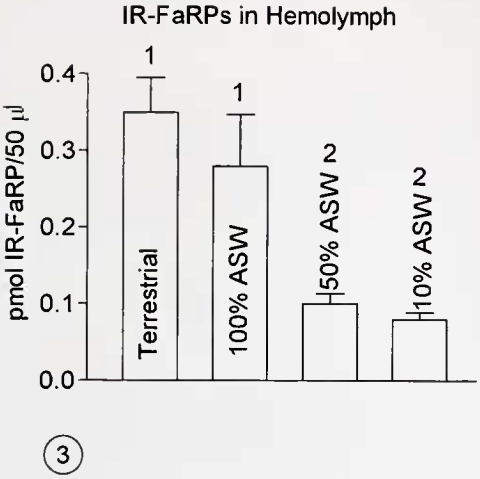
For tissues marked with an asterisk (*), which are very small and difficult to handle for weighing, the IR-FaRP content is expressed as picomole/tissue; in all other tissues the content is expressed as picomole/mg tissue (n = 5; mean ± standard error of mean). The values followed by different letters (a or b) are significantly different ($P < 0.05$, $F = 5.05$) from each other, whereas the values sharing same letters (a or b) are not.

The levels of IR-FaRP in the CNS, hemolymph, and kidney varied with the ambient osmotic conditions. In all three tissues, the levels of IR-FaRPs were correspondingly reduced in lower salinities, and the highest levels were always in terrestrial conditions. The CNS of animals submerged in 10% ASW for 24 h had levels of IR-FaRPs that were 80% lower than those in the terrestrial condition (Fig. 2). In hemolymph, the 10% ASW condition reduced the levels of IR-FaRPs by 77% compared to the terrestrial condition (Fig. 3). In kidney tissues, the 10% ASW condition reduced IR-FaRP levels by 64% compared to the terrestrial condition (Fig. 4).

Immunocytochemistry

Like other dextral gastropods, *M. bidentatus* retains only its right kidney. A whole mount of the kidney reveals IR-staining of axons with varicosities; moreover, such staining and varicosities are restricted to the kidney, and none are present in the surrounding mantle tissue (Fig. 5). Longitudinal sections of the kidney also show localized IR-staining at the periphery (Fig. 6). Some aggregation of varicosities appear as intense staining. No IR-cell bodies were seen in the kidney.





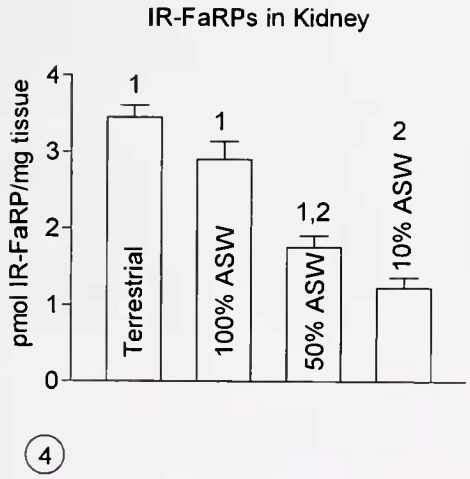


Figure 2. IR-FaRPs in the CNS. Note that the level of IR-FaRPs in the terrestrial condition is higher than that in the three ASW conditions ($P < 0.05$ for 100% ASW and 50% ASW; $P < 0.01$ for 10% ASW. $F = 18.02$) ($n = 5$; mean ± standard error of mean).

Figure 3. IR-FaRPs in the hemolymph. The levels of IR-FaRPs in the terrestrial and 100% ASW conditions are significantly greater than those in 50% and 10% ASW conditions ($P < 0.01$ and $P < 0.001$, respectively. $F = 10.50$) ($n = 5$; mean ± standard error of mean).

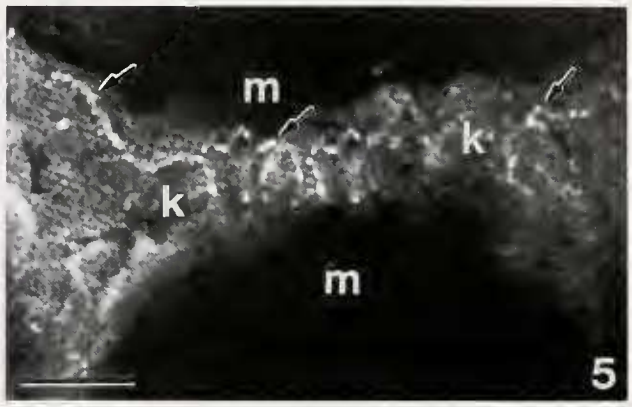


Figure 5. Optical section of the whole mount of the kidney (k) and adjacent mantle (m) tissue showing IR-fibers and varicosities (arrows) in the kidney. Scale bar: 50 µm

Figure 6. Longitudinal section of the kidney showing IR-staining (thin arrows) possibly as innervations to smooth muscles within the kidney tissue and also in the periphery. kl, kidney lumen; mc, mantle cavity. Scale bar: 300 µm..

The distribution of IR-neurons in the CNS is mapped in Figure 7. All commissures, connectives, and major nerves in the CNS display IR-fibers, and all ganglia except the pleurals contain IR-cell bodies. Visceral and right parietal ganglia contain both large (20 ± 5 µm) and small (10 ± 5 µm) IR-neurons, whereas mostly small and only a few large IR-neurons are found in the cerebral and pedal ganglia (Figs. 7–9, 12).

Cerebral ganglia. Two groups of large (20 ± 5 µm) and small (10 ± 5 µm) IR-neurons are present at the anterior and posterior location of the cerebral commissure (Figs. 7–9). The middle part of each cerebral ganglion contains a large (20 ± 5 µm) IR-cell surrounded by small IR-cells (Fig. 9). In the lateral lobes, 2–4 large (20 ± 3 µm) cells and 8–12 small (12 ± 4 µm) IR-cells are seen (Figs. 7, 10).

Figure 4. IR-FaRPs as detected by antiserum S253 in the kidney. The level of IR-FaRPs in the terrestrial condition is significantly ($P < 0.05$. $F = 8.17$) higher than that of 10% ASW ($n = 5$; mean ± standard error of mean).

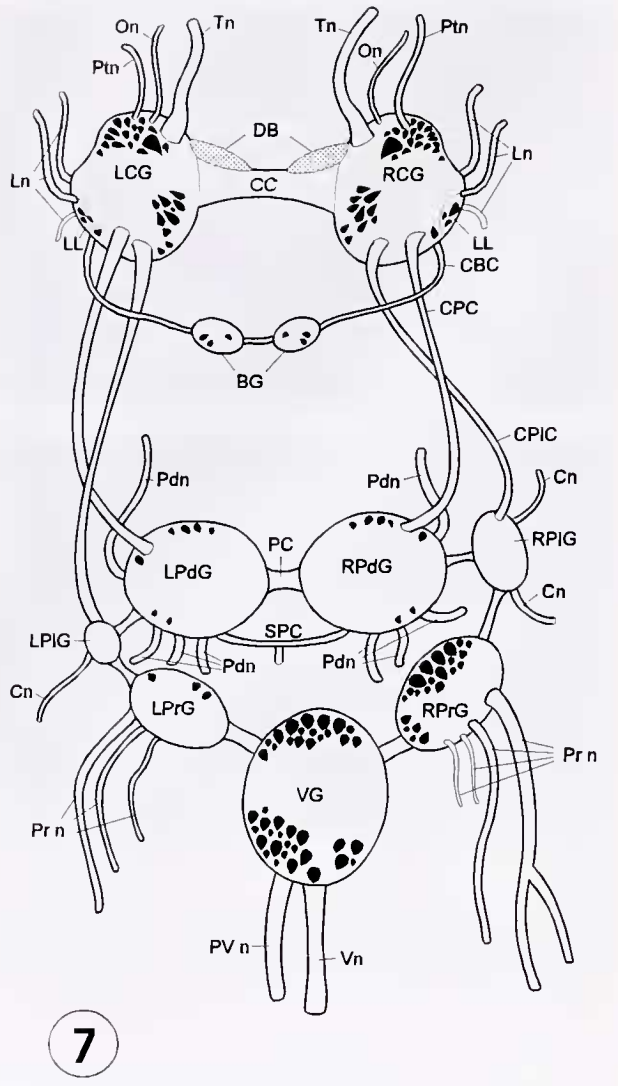


Figure 7. Showing the schematic distribution of the IR-cells in the CNS. BG, buccal ganglia; CBC, cerebro-buccal connective; CC, cerebral commissure; Cn, cutaneous nerve; CPC, cerebro-pedal connective; CPlC, cerebro-pleural connective; DB, dorsal bodies; LCG, left cerebral ganglion; LL, lateral lobe; Ln, labial nerves; LPdG, left pedal ganglion; LPlG, left pleural ganglion; LPrG, left parietal ganglion; On, optic nerve; PC, pedal commissure; Pdn, pedal nerve; Ptn, peritentacular nerve; Prn, parietal nerves; PVn, parieto-visceral nerve; RCG, right cerebral ganglion; RPdG, right pedal ganglion; RPlG, right pleural ganglion; RPrG, right parietal ganglion; SPC, sub-pedal commissure; Tn, tentacular nerve; VG, visceral ganglion; Vn, visceral nerve. Not to scale.

Buccal ganglia. In each buccal ganglion, 3 intermediate sized (18 ± 2 μm) IR-cells, and many IR-fibers and varicosities are seen (Fig. 11).

Pedal ganglia. Groups of 6–10 small IR-neurons are seen at the periphery of each pedal ganglion (Figs. 7, 12).

Parietal and visceral ganglia. More large and small IR-cells were seen in the right parietal and visceral ganglia than in any other ganglion (Figs. 7, 13). The left parietal ganglion contains only small IR-neurons (Fig. 7). The IR-cells are arranged in anterior and posterior groups in both ganglia (Figs. 7, 14). Numerous IR-neurites emanate from the CNS into the surrounding connective tissue and appear to terminate in varicosities (Fig. 15).

Discussion

In both the field and the laboratory, most individuals of *M. bidentatus* were emergent, living under semiterrestrial conditions. But some members of the population were always submerged, suggesting that they crawl in and out of water intermittently. The osmotic concentration of the hemolymph was correlated with that of the adjacent aqueous medium. Moreover, IR-FaRPs are present in the CNS, kidney, and hemolymph, and the levels of these peptides are also correlated with the osmotic concentration of the ambient medium.

Osmoregulation and kidney

When the osmolarity of the external medium changes, the hemolymph osmolarity of *M. bidentatus* also changes due to inward and outward movements of water from its body. Such conditions must be offset by various adjustments: regulation of urine production by its kidney is one of them. The IR-staining of the kidney was seen as a diffuse pattern on the kidney cells, as an intense localized pattern in areas with neural arborizations and varicosities, and in other small areas that are probably bundles of smooth muscles. The smooth muscles of the kidney of the basommatophoran snail *Helisoma duryi* are innervated by FMRFamide-immunoreactive axons (Saleuddin *et al.*, 1992). In this species the *in vitro* contraction of kidney tissues was influenced by FaRPs (unpubl. obs., A.S.M. Saleuddin). The FaRPs are well known for their contractile activity on visceral and smooth muscles in various species. They are also known to regulate salivary glands and ion channels (Bulloch *et al.*, 1988; Green *et al.*, 1994; Price and Greenberg, 1994; Lingueglia *et al.*, 1995). In the pulmonate kidney they are probably involved in regulating smooth muscle contractions for peristalsis and the production of hydrostatic pressure for urine formation, as well as in regulating ion channels for selective secretion and reabsorption of ions from urine.

Osmoregulation and IR-FaRP levels

The CNS and hemolymph displayed the highest and lowest levels of IR-FaRPs, respectively. Organs containing involuntary muscles, such as the reproductive tracts and kidney, have high levels of IR-FaRPs. These organs apparently receive FaRPs through direct innervation by neurons from the CNS. Thus, in these target tissues, the FaRPs may act as neuromodulators or as paracrine agents. Numerous IR-FaRP processes terminating in varicosities in the connective tissue around the CNS may constitute a diffuse

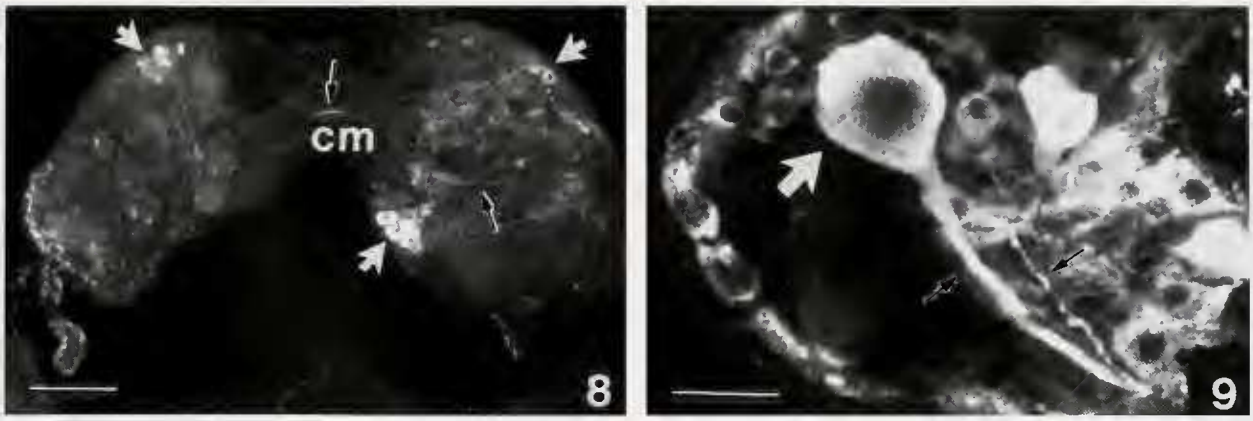


Figure 8. Groups of IR-cells (large arrows) in a cross section of the left and right cerebral ganglia; thin arrows point to IR-material in the commissure (cm) and neuropile. Scale bar: 100 μm.

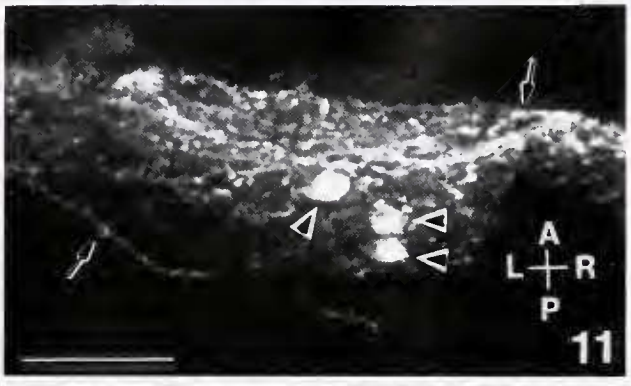
Figure 9. Optical section of the left cerebral ganglion showing a large IR-cell (large arrow) among small IR-FMRFamide cells and their neurites (small arrows). Scale bar: 20 μm.

neurohemal area and account for the hemolymph IR-FaRP levels.

The levels of IR-FaRPs in the CNS, hemolymph, and kidney varied under different osmotic conditions. The IR-FaRP levels were reduced in hypoosmotic conditions compared with those in hyperosmotic or terrestrial conditions. Terrestrial or hyperosmotic salt-water conditions produced threats of desiccation and salt loading in snails, whereas hypoosmotic conditions had the opposite effect. The changes of IR-FaRP levels in the tissues of *M. bidentatus* by osmotic conditions may reflect osmoregulation by the snail. Since IR-FaRP levels increased in the CNS, hemolymph, and kidney under conditions of increased water losses such as in terrestrial or 100% ASW conditions, the FaRPs may have an antidiuretic function. *In vitro* culture of the kidney tissues of *Helisoma* with synthetic FMRFamide increased its intracellular water uptake, and FMRFamide has been suggested to have an antidiuretic role (Saleuddin *et al.*, 1992; Madrid *et al.*, 1994). Antidiuretic and diuretic activities of FMRFamide and GDPFLRFamide respectively have been demonstrated in nephridia of a leech (Salzet *et al.*, 1994). Basommatophoran pulmonates such as *Melampus* contain GDPFLRFamide (unpub. obs., D. A. Price). Both tetra- and hepta-FaRPs are present in the osmoregulatory tissues of *Helisoma* (Madrid *et al.*, 1994). The tetra- and hepta-FaRPs are expressed in exclusive neurons and may have different actions in a target tissue (Greenberg and Price, 1992; Benjamin and Burke, 1994; Price and Greenberg, 1994). The precise mechanism of the tetra- and hepta-FaRP actions in molluscan kidneys awaits future studies. In molluscs, osmoregulation by the kidney appears to be a combined effect of many physiological systems regulated by different hormones. In *Lymnaea stagnalis*, a neuropeptide that stimulates sodium uptake by the skin has been sequenced; furthermore, axons that release immunoreactive sodium-influx-stimulating peptides are present in this snail (De With *et al.*, 1994). In *Aplysia californica*, R15α1 neuropeptide causes water retention, thus having an osmoregulatory role (Weiss *et al.*, 1989). Additionally, in this species various kidney functions are modulated by L10 and LUQ neurons, and the latter neurons are immunoreactive to FMRFamide (Koester and Alevizos, 1989; Giardino *et al.*, 1996). Such systems include increased circulation of hemolymph for ultrafiltration, subsequent secretion and reabsorption of ions and organic matters, and expulsion of final urine (Khan and Saleuddin, 1979). Bioassay studies assessing the effects of FaRPs on kidney functions will enhance understanding of hormonal control of osmoregulation.

Location of IR-FaRP cells

The neurosecretory cells of the CNS of *M. bidentatus* have been described by using histochemical staining (Price, 1977; Ridgway, 1987). The IR-FaRP cells described in this paper are some of these neurosecretory cells; their pattern of distribution is basically similar to that of other basommatophoran pulmonates such as *Lymnaea stagnalis* and *Helisoma duryi* (Schot and Boer, 1982; Buckett *et al.*, 1990; Murphy *et al.*, 1985; Saleuddin *et al.*, 1992). In the cerebral ganglia of *M. bidentatus*, the IR-cells are present in groups located anteriorly and posteriorly. Similar groups are also seen in the cerebral ganglia of *H. duryi* near the endocrine growth-regulating mediodorsal cells and the ovulation-regulating caudodorsal cells. In the latter species, however, an additional large group of small IR-cells is seen in the left cerebral extension; such cells are absent in the corresponding location in *M. bidentatus*. The lateral lobes of the cerebral ganglia of *M. bidentatus* contain several large and small IR-cells, whereas those of *H. duryi* contain only two IR-cells, and those of *L. stagnalis* have none (Saleuddin *et*



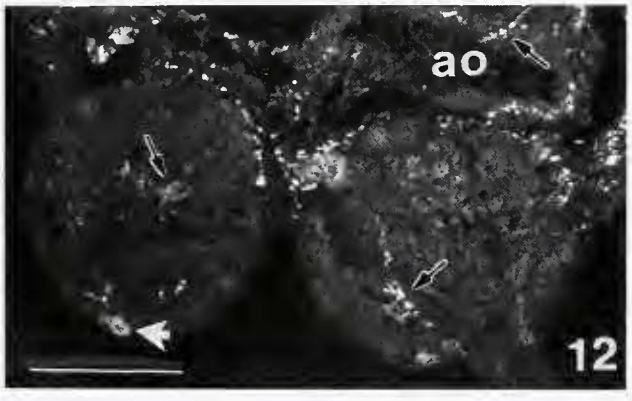
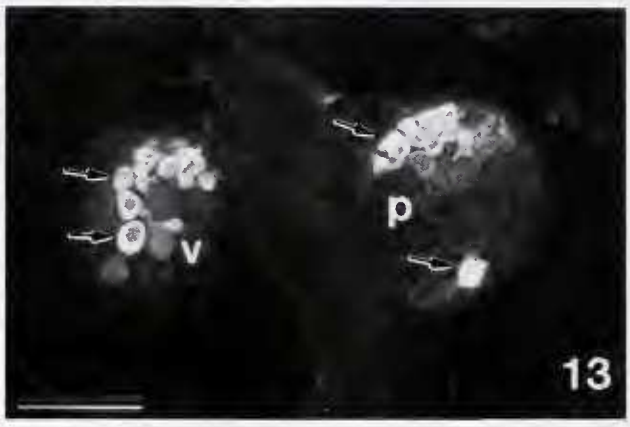


Figure 10. Optical section of the right lateral lobe and a part of right cerebral ganglion (cg) showing large (large arrows) and small (small arrows) IR-cells. Note that the large cells are intensely stained and the small cells are less intensely stained; the cells can be recognized by the lack of staining in their nuclei. Orientation of the tissue is shown by the two axes showing anterior (A) to posterior (P), and left (L) to right (R) sides. Scale bar: 20 μm.

Figure 11. Three cells (arrow heads) and many IR-neurites and varicosities (thin arrows) are shown in the whole mount of right buccal ganglion. Orientation of the tissue is shown by the axes showing anterior (A) to posterior (P), and left (L) to right (R) sides. Scale bar: 50 μm.

Figure 12. Cross section of the pedal ganglia showing a small IR-cell (large arrow) and many IR-neurites (thin arrows). ao, aorta. Scale bar: 250 μm.

al., 1992; Schot and Boer, 1982). The lateral lobes of *L. stagnalis* and *H. duryi* are known to regulate both growth and reproductive centers of the CNS (for review, see Saleuddin *et al.*, 1994; Saleuddin and Ashton, 1996). In the buccal ganglia of *M. bidentatus*, three IR-cells have been seen; in those of *H. duryi* two cells occur; and in those of *H.*



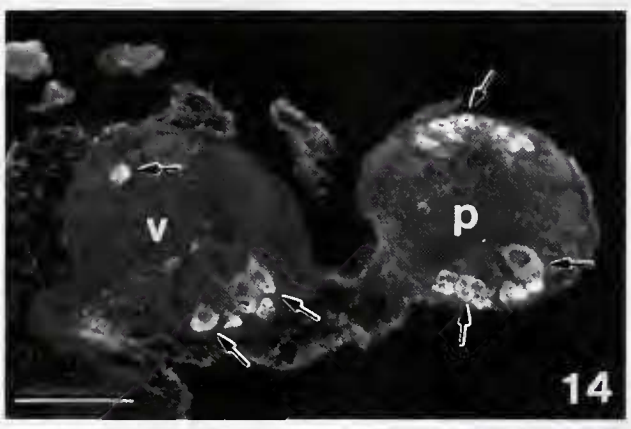


Figure 13. Cross section of the anterior region of the visceral (v) and mid region of the right parietal (p) ganglia showing IR-cells (thin arrows). Scale bar: 50 μm.

Figure 14. Cross section of the mid region of the visceral (v) and posterior region of the right parietal (p) ganglia showing IR-cells (thin arrows). Scale bar: 50 μm.

Figure 15. Whole mount of the cerebral commissure (com) showing extensive IR-neurites and varicosities within the connective in adjacent area (arrows). Orientation of the tissue is shown by the two axes showing anterior (A) to posterior (P), and left (L) to right (R) sides. Scale bar: 50 μm.

trivolvis about 30 smaller cells have been seen (Murphy *et al.*, 1985; Saleuddin *et al.*, 1992). The IR-cells in the parietal and visceral ganglia of *M. bidentatus* are arranged in anterior and posterior groups, which appear to be similar to those in *H. duryi* and *L. stagnalis* (Schot and Boer, 1982; Buckett *et al.*, 1990; Saleuddin *et al.*, 1992). The location of IR-cells in the basommatophoran pulmonates such as *H. duryi* and *L. stagnalis* is close to or within important endocrine centers that regulate growth and reproduction, and the lateral lobes of the cerebral ganglia that regulate both growth and reproduction. Numerous IR-fibers emanate from the CNS and terminate as varicosities in the nearby connective tissue, suggesting that the FaRPs are released into the hemolymph that perfuses the CNS. The FaRPs may also participate in regulating other endocrine centers of the CNS.

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